12.0 MINIMUM PROCEDURAL STANDARDS FOR *IN VITRO* AR TA ASSAYS AND RECOMMENDED SUBSTANCES FOR USE IN VALIDATION STUDIES

12.1 Introduction

Few *in vitro* studies have been published on the ability of substances to act as AR agonists or antagonists. Furthermore, except for the report submitted by Otsuka Pharmaceutical Co. (2001), which evaluated the agonist activity of 65 substances, the number of substances tested for agonist or antagonist activity in each report ranged from one to 22, with most publications based on about ten substances. There are no published guidelines for conducting such studies, and no formal validation studies have been conducted to assess the performance or reliability of *in vitro* AR TA assays. To assist in the development and characterization of these assays, minimum procedural standards and a recommended list of test substances for use in validation studies are provided. The minimal procedural standards and recommended test substances are based on an evaluation of the specific *in vitro* AR TA assays considered in **Sections 6 and 7** of this BRD (**Appendix D**). For the reasons discussed in **Sections 6** and **11**, an assay with endogenous AR and a stably transfected reporter vector containing the *Luc* gene, is recommended as having the highest priority for future validation efforts.

12.2 Minimum Procedural Standards

The minimum procedural standards listed below are recommended for standardized protocols developed for various types of AR TA assays. Adequate procedural details are essential to maximize interlaboratory reproducibility and minimize variation that may contribute to erroneous or nonreproducible results.

12.2.1 Transcriptional Activation of the Reference Androgen

Irrespective of the source of the cell line used, the transcriptional activation-inducing ability of the reference androgen (**Section 12.2.2**) must be demonstrated each time the test is conducted. Consistency in the level of the reporter gene product response induced by the reference androgen is used as a measure of the intralaboratory reproducibility of the assay, and as a criterion for assay acceptance. Since it has been demonstrated in *in vitro* AR TA antagonism assays that the ability to detect a weak antagonist depends on the concentration of the reference androgen, this concentration must be based on the dose response of that androgen in the particular cell line

being used for AR-induced transcriptional activation. It is suggested that the dose should give 70-80% of the maximal response in the cell line. This reference dose can be determined by measuring transcriptional activation in the cell line over a range of concentrations.

12.2.2 Reference Androgen

Similarly to the *in vitro* assays that measured AR binding, where four different reference androgens were used, the same four reference androgens have been used in *in vitro* AR TA assays (**Table 2-1**). For the majority of such studies, DHT has been used as the reference androgen. Since testosterone can be metabolized to DHT in some cell lines (e.g., CV-1, HeLa) used in *in vitro* AR TA studies, most investigators have avoided using it as the reference androgen. However, three investigators have used this substance as the reference androgen. R1881, a potent synthetic androgen, was used by four investigators (five publications) as the reference androgen. Mibolerone was used as the reference androgen by Takeo and Yamashita (2000) for studies involving AR from the rainbow trout. Since most investigators have used DHT as the reference androgen and the issue of DHT binding to a testosterone-estradiol binding globulin (TeBG) is not relevant to *in vitro* AR TA studies, DHT could be used as the reference androgen of choice. However, if a comparison of data between AR binding and AR TA assays is deemed important, it would be more appropriate if the same reference androgen were used for both types of assays. Thus, under these circumstances, R1881 would be recommended for both types of assays as the reference androgen.

12.2.3 Preparation of Test Substances

Test substances must be dissolved in culture medium or in a solvent that is miscible with the medium. For substances not sufficiently water soluble, absolute ethanol or DMSO are proposed as solvents. Preference is given to absolute ethanol since this solvent has been used in most of the studies conducted to date. Other solvents may be used as long as it can be demonstrated that they do not interact, or otherwise interfere, with the test system. A solvent control substance must be included in each assay.

12.2.4 Concentration Range of Test Substances

To minimize effort and costs in screening/testing, and in recognition that adding excessive amounts of a test substance can perturb the test system through physicochemical mechanisms, most testing schemes include a limit dose (i.e., the highest dose that should be tested in the absence of solubility or toxicity constraints). An agreed upon limit dose for *in vitro* AR TA screening assays has not been established. Historically, the highest dose tested in such assays has ranged from 1 to 100 µM, with most tests conducted using a maximum dose level of 100 µM. The EC_{50} values reported for substances tested in various *in vitro* AR TA assays cover eight to nine orders of magnitude (from 20 pM to 8 mM) although the majority of EC_{50} values ranged from 20 pM to 100 nM. Thus, if the *in vitro* AR TA assay is required to detect substances with an EC_{50} that is at least 8 orders of magnitude higher than that of DHT, then the limit dose (unless precluded by chemical properties such as solubility) should be 100 µM. However, if seven orders of magnitude are sufficient for detecting AR agonists, then the limit dose could be 10 µM.

Therefore, for the *in vitro* screening for AR agonists, it is proposed that the limit dose be 100 µM and that a concentration range from 10 pM to 100 µM, in 10-fold increments, be used in each experiment. However, if it is suspected that the test substance binds weakly to the AR, the dose range should extend from 10 nM to 10 mM, in 10-fold increments.

For AR antagonism assays, the weakest AR antagonist, toxaphene, (see **Table 7-2**) had a reported IC_{50} value of 1.935 mM. Therefore, the range of substance concentrations tested in such studies should be from 10 nM to 10 mM.

For relatively insoluble substances, the highest dose should be at the limit of solubility; the concentration range should then decrease in 10-fold increments. Testing at concentrations that result in precipitation in the test medium should be avoided to minimize false positive results associated with the nonspecific interaction of the precipitate with the receptor (Gray et al., 1997).

12.2.5 Solvent and Positive Controls

Concurrent negative and solvent controls and a reference androgen must be included in each experiment. The negative control provides assurance that the solvent does not interact with the test system. The solvent should be tested at the highest concentration that is added with the test substance. The reference androgen in *in vitro* AR TA agonism assays is included to demonstrate the sensitivity of the assay in each experiment for detecting agonist activity and to allow for an assessment of variability in the conduct of the assay across time. A reference androgen for *in vitro* AR TA antagonism assays is required for the assay to function. In addition, to demonstrate the sensitivity of the *in vitro* AR TA antagonism assay, a substance with demonstrated AR antagonism activity (i.e., a positive control) is needed in each experiment. Hydroxyflutamide is suggested as the candidate AR antagonist as this substance has historically been shown to be negative as an agonist but positive as an antagonist.

12.2.6 Within-Test Replicates

Triplicate values should be obtained for each dose tested for each control and test substance.

12.2.7 Dose Spacing

Generally, to obtain a response curve to assess AR-induced transcriptional activation, the concentrations of the reference androgen and the test substances should be spaced by one order of magnitude (i.e., 1 nM, 10 nM, etc.) over the concentration range of interest (1 pM to 100 μM). For antagonists, the concentration range should range from 10 nM to 1 mM. This results in the testing of nine concentrations of each substance for agonists and six concentrations of each substance for antagonism in each test. If the range of doses is reduced due to, for example, insolubility of the test substance at the limit dose, then equivalent spacing (e.g., half-log doses) of the nine or six doses over the smaller dose range should be used.

12.2.8 Data Analysis

Different investigators have used various approaches for analyzing data obtained from *in vitro* AR TA assays. For agonist assays, responses are compared to the concurrent vehicle control while for antagonist assays, treatments are compared to the response induced by the reference androgen alone. Data analysis approaches have varied from a visual inspection of the data only

to more formal statistical approaches using either one- or two-way analysis of variance (ANOVA) (with main effects being treatment or replicates and treatment, respectively) using a general linearized model. In some studies, the induced reporter gene response for each replicate has been converted to a fold induction above the concurrent control level, and means and variances of these data used as the basis for analysis. EC_{50} or IC_{50} values have been calculated using various curve fitting programs. One curve fitting approach was based on a logistic dose response model where the asymptotic minimum and maximum response, the dose that is halfway between the minimum and maximum, and the slope of the line tangent to the logistic curve at this mid-point is determined (see Deslypere et al., 1992; Gaido et al., 1997). Asymptotic standard errors of the parameter estimates are employed to perform two-sided "t" tests.

It would be useful for future validation studies to compare and evaluate the various methods used to analyze *in vitro* AR TA agonist and antagonist data in order to develop standard approaches.

12.2.9 Assay Acceptance Criteria

An *in vitro* AR TA assay testing for agonism activity should be accepted only if the response for the reference androgen occurs within the appropriate confidence limits based on historical data. An *in vitro* AR TA assay testing for antagonism activity should be accepted only if the response for the reference androgen and the positive antagonism control occur within the appropriate confidence limits based on historical data.

12.2.10 Evaluation and Interpretation of Results

A substance is classified as an AR agonist if the assay-specific response (e.g., luciferase activity) is significantly increased above the concurrent control level, as determined by an appropriate statistical test. A substance is classified as an AR antagonist if the substance induces a significant decrease in the ability of the reference androgen to induce TA, as determined by an appropriate statistical test.

12.2.11 Test Report

At a minimum, the test report must include the following information:

Test substance:

- Name, chemical structure, and CASRN, if known;
- Physical nature (solid or liquid), and purity, if known (every attempt should be made to obtain the purity); and
- Physicochemical properties relevant to the study (e.g., solubility, stability, volatility).

Solvent:

- Justification for choice of solvent if other than medium, absolute ethanol, or DMSO;
- Information to demonstrate that the solvent, if other than medium, absolute ethanol, or DMSO, does not affect the sensitivity of the assay.

Androgen receptor:

- Type and source of AR (if from a commercial source, the supplier must be identified);
- Isolation procedure or method for making constructs; and
- Nomenclature and components of the expression and reporter constructs.

Reporter plasmid:

- Type of reporter gene;
- Type and structure of response elements;
- Original plasmid used to make construct; and
- Description and methodology used to make plasmid that is transfected.

Cell line:

- Source of cell line and protocol for maintenance of the cell line;
- Growth parameters of the cell line before initiation of the assay; and
- Method used to transfect cells if transiently transfected cells are used.

Test conditions:

- Rationale for the concentration of the reference androgen used;
- Composition of media and buffers used;
- Concentration range of test substance with justification;

- Volume of solvent used to dissolve test substance and volume of test substance added;
- Incubation time and temperature;
- Type and composition of metabolic activation system, if added;
- Concentration range of positive and solvent controls;
- Method used to lyse cells after incubation;
- Method used to measure transcriptional activation;
- Methods used to determine fold induction, EC_{50} value for agonism studies, or IC_{50} value for antagonism studies; and
- Statistical methods used.

Results:

- Extent of precipitation of test substance;
- Reporter response for each replicate at each dose for all test substances, including confidence levels or other measure of intradose repeatability;
- Calculated EC_{50} value for agonism studies or IC_{50} value for antagonism studies, and confidence limits, if calculated, for the reference androgen (agonism studies), positive control (antagonism studies), and test substance; and
- Fold increase above control for each concentration.

Discussion of the results:

- Historical fold increases in activity and EC_{50} values for reference androgen (agonism), including ranges, means, and standard deviations; and
- Reproducibility of IC_{50} value of positive control antagonist compared to historical data.

Conclusion:

- Classification of test substance with regard to *in vitro* AR agonist or antagonist activity.

12.2.12 Replicate Studies

Generally, replicate studies are not mandated for screening assays. However, in situations where questionable data are obtained (i.e., the fold increase is marginal, the EC_{50} or IC_{50} value is not

well defined, the call is equivocal, the test shows excess variability), repeat tests to clarify the results of the primary test would be prudent.

12.3 Standardization of *In Vitro* AR TA Assays for Validation

Appendix B provides *in vitro* AR TA assay protocols submitted by four investigators. The assay protocols, as titled by the investigators, are:

- Protocol for CV1 + hAR + Luciferase Assay, as provided by Dr. Elizabeth M. Wilson, Departments of Pediatrics and of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC, USA.
- Protocol for CHO Cells + hAR + Luciferase Assay as provided by Dr. Anne Marie Vinggaard, Institute of Food Safety and Toxicology, Danish Veterinary and Food Administration, Soborg, Denmark.
- Protocol for HepG2 Cells + Receptor + Reporter and/or β-gal Plasmids for Use in Steroid Hormone Receptor Assays, as provided by Dr. Kevin Gaido, CIIT Centers for Health Research, Research Triangle Park, NC, USA.
- Protocol for Yeast-Based Androgen Receptor Assay, as provided by Dr. Kevin Gaido, CIIT Centers for Health Research, Research Triangle Park, NC, USA.
- Development of New Reporter Gene Assay Systems for Screening Endocrine Disrupters, as provided by Drs. Mitsuru Iida and Teruhisa Kato, Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan
- Development of Stably Transfected Cell Lines to Screen Endocrine Disrupters, as provided by Drs. Mitsuru Iida and Teruhisa Kato, Otsuka Pharmaceutical Co. Ltd., Tokushima, Japan.

Inspection of these protocols provides a perspective on how various *in vitro* AR TA assays are conducted by different investigators. These protocols provide a basis for developing a more general protocol, one that takes into account the recommended minimum procedural standards provided in **Section 12.2**. Prior to developing that protocol, the protocols in **Appendix B** need to be reviewed for completeness and adequacy for their intended purpose.

One of the difficulties in recommending one of the mammalian cell assays as a screening test method for detecting substances with AR agonist or antagonist activity is the issue surrounding

the patents that exist for the AR gene sequence and the technology for transfecting this receptor into mammalian cells (**Section 11**). As a result, it has been difficult to further develop these *in vitro* assays as AR TA test methods. One approach to overcoming this restraint has been the use of cell lines that harbor an endogenous AR gene that then requires transfection or transduction of the reporter construct only. This has been the approach used by Gray and coworkers with the MDA-MB-453 cell line (Hartig et al., 2002; Wilson et al., 2002). Subsequently, these investigators developed a variant of this cell line (MDA-MB453-kb2) that was stably transfected with a luciferase reporter construct. A stably transfected cell line has also been developed by transfecting an AR expression vector and a reporter gene construct that also carry antibiotic resistant genes for selection (Terouanne et al., 2000). Neither of these cell lines has been validated for their intended use and both have associated limitations (**Table 6-2**). Nevertheless, at the present time, these cell lines or ones similar to them that might be developed in the future, have the greatest potential for use as an *in vitro* AR TA screening assay. One of the difficulties with the MDA-MB453-kb2 cell line is the use of the MMTV promoter. This response element contains sequences to which both the AR and GR can bind. Thus, the presence of the GR can alter the transcriptional activation responsiveness of the cell if the test substance binds to it as well as to the AR (e.g., medroxyprogesterone acetate). This is a problem with any cell line that has a GR that is transfected with reporter plasmid that contains the MMTV-Luc. However, the AR activity can be distinguished from that of GR with the use of selective competitors (Wilson et al, 2002).

12.4 List of Recommended Substances for Validation of *In Vitro* AR TA Assays

Tables 12-1 and **12-2** provide lists of recommended substances to be used in the assessment of the reliability and comparative performance of *in vitro* AR TA agonist and antagonist assays, respectively. A number of factors were considered in developing the list for AR agonist studies, including the number of times the substance had been tested in any assay, the median EC_{50} value (when available) of the substance in all the assays in which it was tested (see **Table 7-1**), the fold increase in response above the control substance, and whether it had been recommended for testing in the AR binding BRD. The latter was considered since it would be informative to assess whether a substance was positive for AR binding but did not elicit a positive transcriptional activation response or vice-versa. For antagonists, the median IC_{50}, if available,

and the fold decrease in transcriptional activation compared to the reference androgen was used. Selection of the substances was based on the availability and concordance of multiple test results among the *in vitro* AR TA assays considered in **Appendix E**. Because quantitative data was available for only a few substances, consideration was given to qualitative responses (i.e., positive, weak positive, or negative). Methoxychlor (Gaido et al., 2000) and vinclozolin (Wilson et al., 2002) have been included in the substances to be tested for antagonism even though they have to be activated in the cell as they have been shown be active in HepG2 and MDA-MB-453-kb2 cells, respectively. It might be more appropriate to use the metabolites of these compounds for testing but they are not available from a commercial source.

In a validation study, it is important to include substances that cover the range of possible responses and, therefore, this list includes substances in each category. The variability in the numbers of strong, weak, and negative substances in each list reflects the available database.

Table 12-1 List of Substances Recommended for Validation of *In Vitro* AR TA Assays for Agonism

Substance	CASRN	Qualitative Response for AR Agonism[a]	EC_{50} Value (μM)[b]	RBA[c]
Levonorgestrel	797-63-7	Positive (3)	0.000984	9.25
Methyltestosterone	58-18-4	Positive (2)	0.000812	
Androstenedione	63-05-8	Positive (3)	0.00153	1.03
Testosterone	58-22-0	Positive (8)	0.00245	29.2
Mifepristone	84371-65-3	Positive (4/5)*	0.0136	
Cortisol	50-23-7	Positive (3/5)*	0.043	HTD-1000 μM
Estrone	53-16-7	Positive (2)	0.0551	0.1
17β-Estradiol	50-28-2	Positive (11)	0.0861	1.9
Progesterone	57-83-0	Positive (6/8)*	2.6	3.05
Hydroxyflutamide	52806-53-8	Positive (5/6)	41.5	
Spironolactone	52-01-7	Positive (2)		33.8
Medroxyprogesterone acetate	71-58-9	Positive (4)		11.6
Cyproterone acetate	427-51-0	Positive (5)		3.0
Fluoxymestrone	76-43-7	Positive (1)		0.3
Linuron	330-55-2	Positive (1)		0.0055
Bicalutamide	90357-06-5	Weak (2)		
Fenitrothion	122-14-5	Weak (2)		
Nilutamide	63612-50-0	Weak (1)		
Atrazine	1912-24-9	Negative (1)		0.0018
Corticosterone	50-22-6	Negative (1)		0.000068
o,p′-DDT	789-02-6	Negative (2)		0.0045
p,p′-DDT	50-29-3	Negative (1)		0.0013
Procymidone	320809-16-8	Negative (1)		0.000068
Vinclozolin	50471-44-8	Negative (1)		0.018
Diethylstilbestrol	56-53-1	Negative (3)		0.010
Kepone	143-50-0	Negative (2)		0.00075
Methoxychlor	72-43-5	Negative (2)		0.00054
Flutamide	13311-84-7	Negative (3)		

[a]Numbers in parentheses refer to the number of different agonism assays in which the substance was tested. These counts exclude the cell proliferation assay and the assays using rainbow trout and mouse ARs.
[b]EC_{50} values are medians of the EC_{50} values presented in **Table 7-1.**
[c]RBA = Relative binding affinity reported only for substances recommended for use in validating *in vitro* AR binding assays (NIEHS, 2002). HTD = Highest tested dose.
*Number of assays in which the substance was positive compared to the number of assays in which it was tested.

Table 12-2 List of Substances Recommended for Validation of *In Vitro* AR TA Assays for Antagonism

Substance	CASRN	Qualitative Response for AR Antagonism[a]	IC_{50} Value (μM)[b]	RBA[c]
Mifepristone	84371-65-3	Positive (2)	0.05	
Hydroxyflutamide	52806-53-8	Positive (9)	0.1	
Cyproterone acetate	427-51-0	Positive (5)	0.1	3.0
Nilutamide	63612-50-0	Positive (2)	0.15	
Spironolactone	52-01-7	Positive (2/3)**	0.254	33.8
Vinclozolin	50471-44-8	Positive (3)	0.275	0.018
Diethylstilbestrol	56-53-1	Positive (2)	0.36	0.010
Bicalutamide	90357-06-5	Positive (4)	0.625	
p,p'-DDE*	72-55-9	Positive (5/6)**	3	0.016
Linuron	330-55-2	Positive (2)	5	0.0055
Procymidone	320809-16-8	Positive (2)	7.5	0.000068
Flutamide	13311-84-7	Positive (4)	115	
17β-Estradiol	50-28-2	Positive (4)	0.5	1.9
Medroxyprogesterone acetate	71-58-9	Positive (1)		11.6
Fenitrothion	122-14-5	Positive (2)		
o,p'-DDT	789-02-6	Positive (2)		0.0045
p,p'-DDT	50-29-3	Positive (2)		0.0013
Methoxychlor	72-43-5	Positive (2)		0.00054
Progesterone	57-83-0	Positive (3)	0.3	3.05
Kepone	143-50-0	Equivocal (1/2)**	6.9	0.00075
Testosterone	58-22-0	Negative (1)		29.2
Atrazine	1912-24-9	Negative (1)		0.0018
Fluoxymestrone	76-43-7	Negative (1)		0.3
Lindane*	58-89-9	Negative (2)		

[a]Numbers in parentheses refer to the number of different antagonism assays in which the substance was tested. These counts exclude the cell proliferation assay and the assays using rainbow trout and mouse ARs.
[b]IC_{50} values are medians of the IC_{50} values in **Table 7-2**.
[c]RBA = Relative binding affinity reported only for substances recommended for use in validating *in vitro* AR binding assays (NIEHS, 2002).
*Substances NOT recommended to be tested in *in vitro* AR TA assays for agonism (**Table 12-1**).
**Number of assays in which the substance was positive compared to the number of assays in which it was tested.

12.5 Summary and Conclusions

Currently, there are no published guidelines for conducting *in vitro* AR TA studies, and no formal validation studies have been conducted to assess the reliability or performance of the currently available assays. To support the further development and characterization of *in vitro* AR TA agonism and antagonism assays, minimum procedural standards for such assays and a recommended list of test substances for use in validation studies are provided. The minimum procedural standards and recommended test substances are based on an evaluation of the *in vitro* AR TA assays considered in **Sections 6 and 7** of this BRD. It is recommended that a mammalian cell assay with an endogenous AR and stably transfected reporter gene, as well as a stably transfected plasmid containing β-galactosidase to monitor toxicity be evaluated.

The minimum procedural standards include the following: methods for determining the ability of the reference androgen to induce transcriptional activation; methods for establishing a stable cell line; the concentration range of the test substance (including the limit dose) to test for agonists and antagonists; the use of negative, solvent, and positive controls; the number of replicates to use; dose spacing; data analysis; assay acceptance criteria; evaluation and interpretation of results; minimal information to include in the test report; and the potential need for replicate studies. These minimum procedural standards are provided to ensure that *in vitro* AR TA studies will be conducted in such a manner as to allow the results to be understandable and comparable among procedures.

Six submitted *in vitro* AR TA assay protocols developed by experts in the field are provided in **Appendix B**. Inspection of these protocols provides a perspective on how various *in vitro* AR TA assays are conducted by different investigators, and for developing a more general protocol, one that takes into account the recommended minimum procedural standards. Prior to developing that protocol, the appended protocols need to be evaluated for completeness and adequacy for their intended purpose.

A number of factors were considered in developing a list of substances to be used in validation efforts, including the EC_{50} and IC_{50} value of the substance in all of the assays in which it has

been tested. Because the number of substances with replicate quantitative agonist or antagonist data was insufficient to generate the desired number of substances for consideration, selection of most substances was based on results obtained in a single assay by a single investigator. The selected substances were sorted according to whether they were positive, weak positive, or negative in at least one *in vitro* AR TA assay.

It is anticipated that this BRD and the guidance it provides will help to stimulate validation efforts for *in vitro* AR TA assays.